

# Platelet CD40 Exacerbates Atherosclerosis by Transcellular Activation of Endothelial Cells and Leukocytes

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# Platelet CD40 Exacerbates Atherosclerosis by Transcellular Activation of Endothelial Cells and Leukocytes

Norbert Gerdes, Tom Seijkens, Dirk Lievens, Marijke J.E. Kuijpers, Holger Winkels, Delia Projahn, Helene Hartwig, Linda Beckers, Remco T.A. Megens, Louis Boon, Randolph J. Noelle, Oliver Soehnlein, Johan W.M. Heemskerk, Christian Weber, Esther Lutgens

**Objective**—Beyond their eminent role in hemostasis and thrombosis, platelets are recognized as mediators of inflammation. Platelet cluster of differentiation 40 (CD40) ligand (CD40L and CD154) plays a key role in mediating platelet-induced inflammation in atherosclerosis. CD40, the receptor for CD40L, is present on platelets; however, the role of CD40 on this cell type is until now undefined.

**Approach and Results**—We found that in both mice and humans, platelet CD40 mediates the formation of platelet–leukocyte aggregates and the release of chemokine (C-X-C motif) ligand 4. Leukocytes were also less prone to adhere to CD40-deficient thrombi. However, platelet CD40 was not involved in platelet aggregation. Activated platelets isolated from *Cd40*<sup>−/−</sup>*Apoe*<sup>−/−</sup> mice adhered less to the endothelium upon injection into *Apoe*<sup>−/−</sup> mice when compared with CD40-sufficient platelets. Furthermore, lack of CD40 on injected platelets led to reduced leukocyte recruitment to the carotid artery as assayed by intravital microscopy. This was accompanied by a decrease in endothelial vascular cell adhesion molecule-1, platelet endothelial cell adhesion molecule, VE-cadherin, and P-selectin expression. To investigate the effect of platelet CD40 in atherosclerosis, *Apoe*<sup>−/−</sup> mice received thrombin-activated *Apoe*<sup>−/−</sup> or *Cd40*<sup>−/−</sup>*Apoe*<sup>−/−</sup> platelets every 5 days for 12 weeks, starting at the age of 17 weeks, when atherosclerotic plaques had already formed. When compared with mice that received *Apoe*<sup>−/−</sup> platelets, those receiving *Cd40*<sup>−/−</sup>*Apoe*<sup>−/−</sup> platelets exhibited a >2-fold reduction in atherosclerosis. Plaques of mice receiving CD40-deficient platelets were less advanced, contained less macrophages, neutrophils, and collagen, and displayed smaller lipid cores.

**Conclusions**—Platelet CD40 plays a crucial role in inflammation by stimulating leukocyte activation and recruitment and activation of endothelial cells, thereby promoting atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2016;36:482–490. DOI: 10.1161/ATVBAHA.115.307074.)

**Key Words:** atherosclerosis ■ blood platelets ■ CD40 ligand ■ immune system ■ leukocytes

Patients with cardiovascular disease display enhanced platelet activity, resulting in a prothrombotic state of the vasculature, and an increased susceptibility to (recurrent) cardiovascular events.<sup>1</sup> However, platelet activity not only figures thrombosis and hemostasis but also transforms the platelet into a protagonist of inflammation, and thereby a driver of atherosclerosis.<sup>2</sup> On activation and consequent granule release, platelets enrich the inflammatory milieu, activate the endothelium, and promote leukocyte recruitment. Platelet-derived chemokines (eg, chemokine (C-C motif) ligand 5, chemokine (C-C motif) ligand 2, and chemokine (C-X-C motif) ligand 4) stimulate the recruitment of leukocytes, whereas

platelet-secreted mediators, such as soluble cluster of differentiation (CD) 40L (sCD40L), serotonin, interleukin-1β (IL-1β), can activate endothelial cells (ECs) and leukocytes.<sup>3–7</sup> In parallel to the release of inflammatory mediators, platelets express a broad range of surface molecules that mediate platelet–leukocyte and platelet–endothelium interactions, including P-selectin,<sup>8</sup> glycoproteins (GP Iba, GP VI, and αIIbβ3),<sup>9,10</sup> toll-like receptors (eg, toll-like receptor-2 and toll-like receptor-4),<sup>11</sup> and costimulatory molecules.<sup>2,4,12,13</sup>

Costimulatory molecules are originally known for their role as key mediators fine-tuning the adaptive immune response. Although the expression was classically ascribed to lymphocytes

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## Nonstandard Abbreviations and Acronyms

<b>CD</b>	cluster of differentiation
<b>EC</b>	endothelial cell
<b>IL</b>	interleukin
<b>(s)CD40L</b>	(soluble) CD40 ligand
<b>VCAM</b>	vascular cell adhesion molecule

and antigen-presenting cells, numerous studies demonstrated the presence of costimulatory molecules on a plethora of cell types, including platelets, thus implicating these cell types in the inflammatory process.<sup>14,15</sup> An important costimulatory dyad in cardiovascular disease is CD40 and its ligand CD40L (CD154).<sup>14</sup> Apolipoprotein E (*Apoe*<sup>-/-</sup>) and Low-density lipoprotein receptor (*Ldlr*<sup>-/-</sup>)–deficient mice exhibit a profound decrease in atherosclerosis when the CD40-CD40L signaling pathway is disrupted. In addition, these plaques contain few inflammatory cells and are rich in extracellular matrix.<sup>16–20</sup>

Both, CD40 and CD40L, are found in human atheroma and their expression increases with plaque progression.<sup>21</sup> Upregulation of platelet CD40L predicts a poor clinical outcome after stroke,<sup>22</sup> and both platelet CD40L levels and monocyte CD40 levels are increased in hyperlipidemic patients.<sup>23</sup> Moreover, serum levels of sCD40L, a cleavage product of CD40L, associates with (recurrent) cardiovascular disease in many studies, such as the c7E3 Fab Antiplatelet Therapy in Unstable Refractory Angina (CAPTURE) trial,<sup>24</sup> the Women's health study,<sup>25</sup> and others.<sup>26–28</sup> Although other clinical trials fail to show a correlation between sCD40L levels and cardiovascular disease<sup>29,30</sup> and in an acute myocardial infarction model in humans,<sup>31</sup> sCD40L is still correlated with other biomarkers of inflammation.<sup>32</sup> Moreover, determination of sCD40L in plasma is challenging, and differences in plasma preparation protocols might mask the validity of sCD40L as a biomarker in atherosclerosis.<sup>33–35</sup>

In mice, we have demonstrated that platelet CD40L plays a crucial role in initiation and progression of atherosclerosis by facilitating platelet–leukocyte aggregate formation and promoting adherence of platelets and leukocytes to the endothelium.<sup>36</sup> However, platelets deficient in CD40L form less stable platelet aggregates<sup>37</sup> and pose an increased risk for thromboembolic events.<sup>38</sup> In addition, we recently showed that CD40L-deficient platelets prevent GP VI–dependent dense thrombus formation on collagen or atherosclerotic plaque material.<sup>39</sup> Conversely, addition of CD40L enhanced GP VI–induced platelet aggregation via integrin  $\alpha$ IIB $\beta$ 3 and phosphatidylinositol-4,5-bisphosphate 3-kinase, but independent of inhibitor of nuclear factor  $\kappa$ -B kinase subunit  $\alpha$ /nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells.<sup>39</sup>

Interestingly, platelets also express substantial levels of the alleged counter-receptor for CD40L, ie, CD40,<sup>40,41</sup> but its role has only been summarily described and suggests a role in thrombus formation.<sup>37,39</sup> Here, we report the participation of platelet CD40 in inflammation mediating EC and leukocyte activation and driving atherosclerosis, whereas it plays a minor role in platelet–platelet interactions.

## Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

## Results

## Deficiency of Platelet CD40 Does Not Impair Platelet Activation

To evaluate the effect of platelet CD40 deficiency on platelet activation, *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> platelets were activated with different concentrations of thrombin, convulxin, and ADP, potent platelet receptor agonists. Corroborating our previous studies, no differences in P-selectin or  $\alpha$ IIB $\beta$ 3 integrin activation were detected between *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> platelets in any of the experimental conditions (Figure 1A and 1B in the online-only Data Supplement).<sup>39</sup> In addition, collagen- or ADP-induced platelet aggregation in whole blood was not affected by deficiency of CD40 (Figure 1A and 1B). However, supernatants of thrombin-activated platelets displayed a significant decrease in chemokine (C-X-C motif) ligand 4, identifying platelet CD40 as a prominent mediator of inflammation rather than a regulator of thrombosis (Figure 1C). Notably, platelets did not release IL-1 $\beta$  (Figure 1C in the online-only Data Supplement).

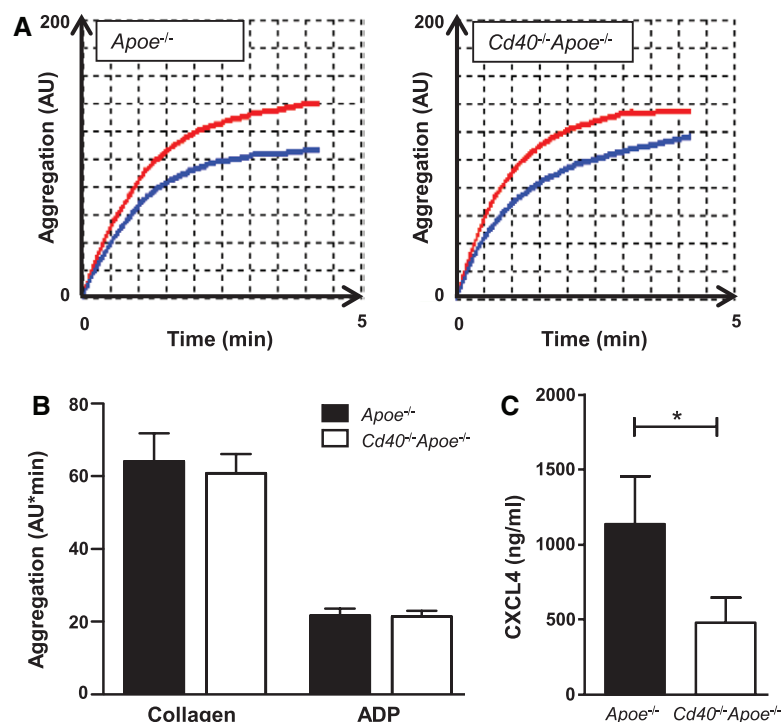
Similar results were found in human platelets, in which CD40 was constitutively present and detectable on the surface on stimulation although overall expression level appeared low (Figure 1I in the online-only Data Supplement). When platelets were activated with an agonistic anti-CD40 antibody (clone G28.5) in combination with ADP or collagen, platelet aggregation was unchanged (Figure 1IIA in the online-only Data Supplement). Corroborating the results obtained with murine platelets, CD40-activated platelets showed an increased release of chemokine (C-X-C motif) ligand 4 (Figure 1IIB in the online-only Data Supplement).

## Platelet CD40 Modulates the Formation of Platelet–Leukocyte Aggregates

In the absence of platelet CD40, the formation of CD45<sup>+</sup>CD41<sup>+</sup> platelet–leukocyte aggregate was significantly impaired (Figure 2A and 2B). In particular, lack of CD40 on platelets attenuated platelet interactions with monocytes (CD11b<sup>+</sup>), dendritic cells (CD11c<sup>+</sup>), and neutrophils (Ly6G<sup>+</sup>), but not with T cells (CD3<sup>+</sup>; Figure 2C–2F). Cytokine levels were measured in the supernatants of platelet–splenocyte cocultures and showed a profound reduction in IL-1 $\beta$  levels, whereas transforming growth factor- $\beta$  release was unaffected (Figure 2G and 2H). Of note, IL-1 $\beta$  released into the supernatant of these coculture systems seems to derive mostly from leukocytes as platelets alone did not secrete appreciable amounts of this cytokine, even on stimulation (Figure 1C in the online-only Data Supplement). The role of platelet CD40 in platelet–leukocyte aggregate formation was also confirmed in human platelets and leukocytes where activation of CD40 increased the amount of CD45<sup>+</sup>CD41<sup>+</sup> aggregates (Figure 1IIC in the online-only Data Supplement).

## CD40-Deficient Platelet Aggregates Show Impaired Leukocyte Recruitment

Because platelets are first responders in vascular injury and thrombosis-promoting leukocyte recruitment into inflamed tissues,<sup>4,42</sup> we used an ex vivo model of collagen-induced



**Figure 1.** Cluster of differentiation 40 (CD40)-deficient platelets exhibit normal platelet activation. **A** and **B**, Platelet aggregation measured by multiple electrode platelet aggregometry (Multiplate). Platelet aggregation was triggered by collagen (4  $\mu$ g/mL) or ADP (4  $\mu$ mol/L) in hirudin-anticoagulated blood from *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice. Two independent aggregation curves (red and blue) were obtained by duplicate sensors located in each test cell. **A**, Representative measurements of collagen-triggered aggregation. **B**, Mean values of the 2 determinations are expressed as area under the curve (n=5). **C**, Chemokine (C-X-C motif) ligand 4 levels measured by ELISA in the supernatants from thrombin-activated (0.5 nmol/L, 15 min) platelets isolated from *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice (n=5 vs 6) \**P*<0.05.

thrombus formation to study platelet-mediated recruitment of leukocytes using platelets from wild-type, *Cd40*<sup>-/-</sup>, *Cd40*<sup>-/-</sup>, and P-selectin-deficient (*Selp*<sup>-/-</sup>) mice. In contrast to abundant leukocyte adhesion to thrombi consisting of *Apoe*<sup>-/-</sup> platelets, deficiency of platelet CD40L, CD40, or P-selectin resulted in a significant decrease of adherent leukocytes in the thrombus (Figure 3A and 3B). Furthermore, when thrombi from *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets were treated with a blocking anti-P-selectin antibody, leukocyte recruitment was almost completely prevented. In agreement with this, *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> thrombi treated with a blocking anti-P-selectin antibody or *Selp*<sup>-/-</sup>*Apoe*<sup>-/-</sup> thrombi treated with an anti-CD40L antibody also resulted in near absence of leukocyte recruitment, suggesting an additive effect (Figure 3A). Of note, platelets of all genotypes did not differ in the extent of thrombus formation (Figure 3C). Two-photon microscopy disclosed that 80% of the attracted leukocytes were Ly6G<sup>+</sup> neutrophils, whereas only 20% were CD11b<sup>+</sup>Ly6G<sup>-</sup> monocytes (Figure 3D–3F). No differences in leukocyte subset distribution could be detected between the different groups (data not shown). These results suggest that thrombi dynamically recruit leukocytes (predominantly Ly6G<sup>+</sup> neutrophils) to a site of injury through intercellular interactions that are mediated by CD40, CD40L, and P-selectin, thus potentially accelerating the progression of atherosclerotic plaques.

### Platelet CD40 Facilitates Platelet–Endothelium–Leukocyte Interactions

When *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets were injected, they showed less abundant adhesion to the endothelium in vivo (Figure 4A and 4B). Moreover, ex vivo stimulation of EC with either activated *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets revealed that also other adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, platelet EC adhesion molecule, VE-cadherin,

and P-selectin, were significantly decreased in the absence of platelet CD40 (Figure 4I).

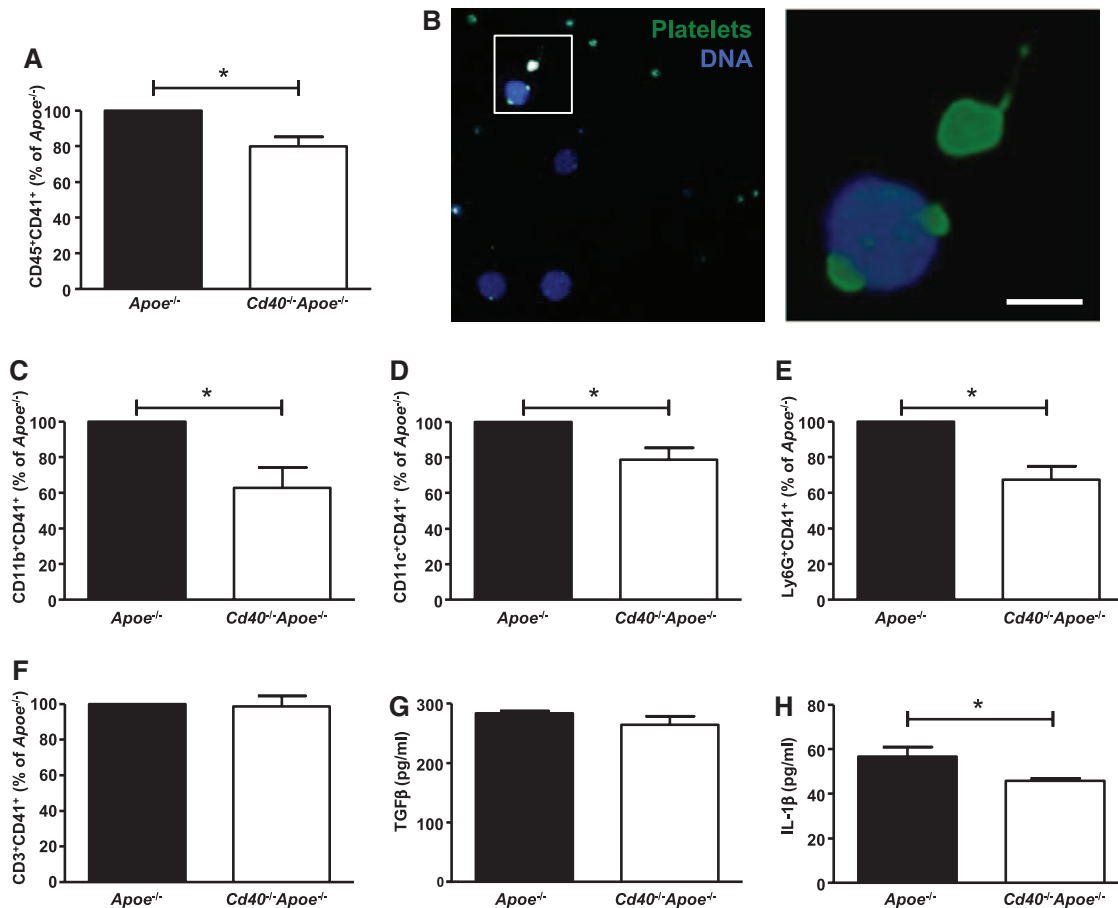
To investigate the effects of recurrent platelet activation on leukocyte adhesion to the carotid artery was analyzed in *Apoe*<sup>-/-</sup> mice that were injected with activated platelets for 4 weeks. In mice that received *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets, leukocyte adhesion was significantly reduced (Figure 4C and 4D) because of a reduction in Gr1<sup>+</sup> inflammatory monocytes and neutrophils (Figure 4E and 4F). In addition, immobilization of anti-VCAM-1-labeled microbeads was significantly reduced in mice injected with *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets (Figure 4G and 4H), suggesting limited endothelial activation and VCAM-1 expression as a potential cause for decreased leukocyte recruitment on injection of *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets.

To determine whether VCAM-1 and IL-1 $\beta$  are involved in platelet-induced leukocyte adhesion, we performed flow chamber experiments in which ECs were cultured and primed with either *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> or *Apoe*<sup>-/-</sup> platelets. Subsequently, the ECs were incubated with anti-VCAM-1, anti-IL-1 $\beta$ , or control antibodies followed by perfusion of leukocytes and their adhesion was monitored. Supporting the in vivo results, priming with CD40-deficient platelets limited platelet-induced leukocyte interactions with ECs (Figure 4J). Moreover, blocking either VCAM-1 or IL-1 $\beta$  potentially reduced platelet-induced leukocyte adhesion indicating a prominent role for these mediators in platelet-modulated leukocyte extravasation (Figure 4K and 4L). Interestingly, when priming with CD40-deficient platelets, anti-VCAM-1 antibody treatment even further limited leukocyte adhesion (Figure 4K).

### Platelet CD40 Promotes Atherosclerosis Progression

To address the role of platelet CD40 in atherosclerosis in vivo, injections of activated platelets in *Apoe*<sup>-/-</sup> mice started at the





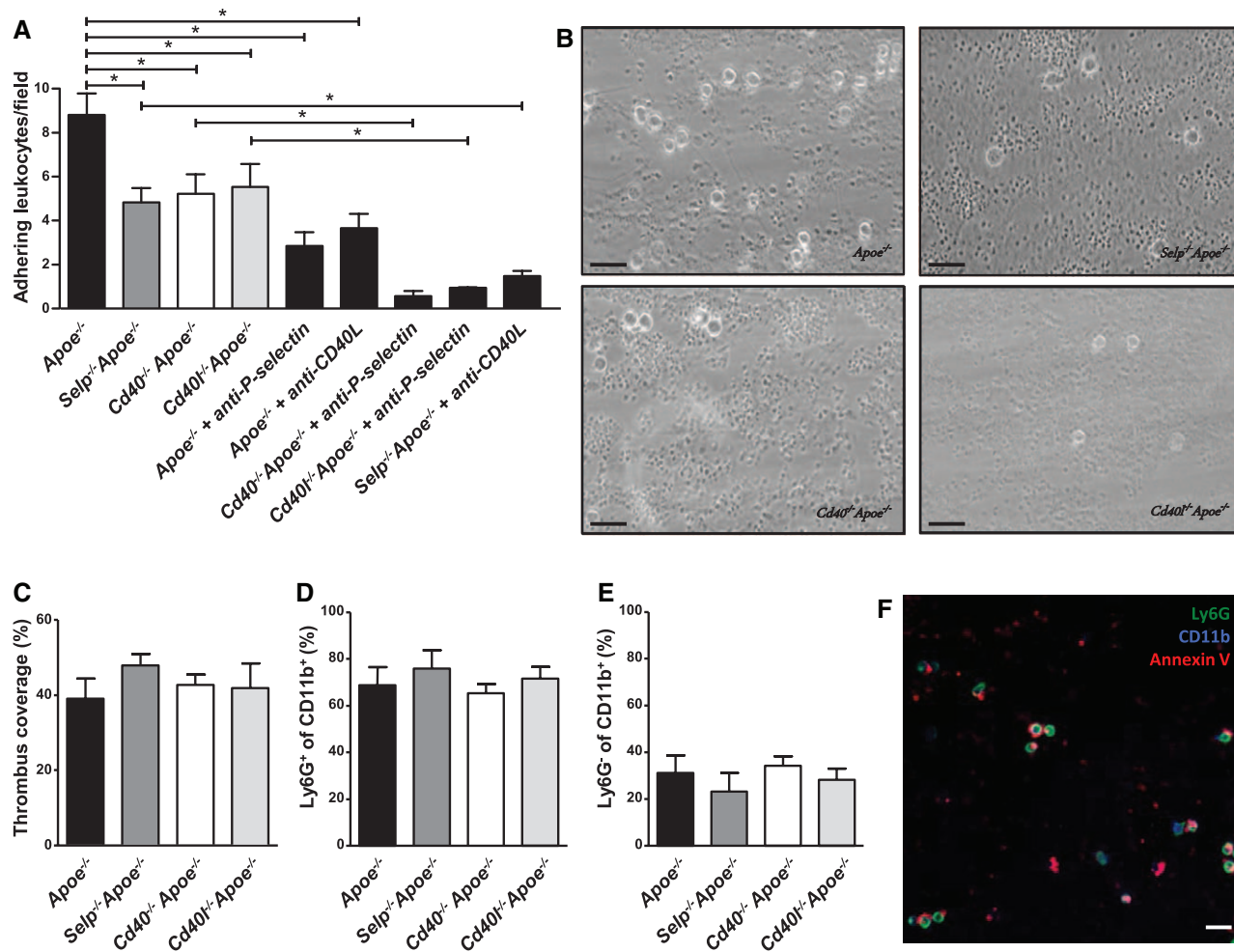
**Figure 2.** Deficiency of platelet cluster of differentiation 40 (CD40) obstructs platelet-leukocyte aggregation (PLA). Platelet-leukocyte aggregation through coincubation of activated platelets isolated from *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice cultured with *Apoe*<sup>-/-</sup> splenocytes, measured by flow cytometry. PLA formation is expressed relative to *Apoe*<sup>-/-</sup> platelets. **A**, Platelets conjugated to CD45<sup>+</sup> leukocytes. **B**, In vitro 2-photon microscopic detection of PLA from *Apoe*<sup>-/-</sup> platelets cultured with *Apoe*<sup>-/-</sup> splenocytes. Overview (left) and higher magnification (right) of the indicated area. Leukocytes were labeled with DAPI (4',6-diamidino-2-phenylindole; blue) and platelets with calcein (green). Scale bar, 5 μm. **C–F**, Flow cytometry analysis of CD41<sup>+</sup> platelets conjugated with (C) CD11b<sup>+</sup> monocytes, (D) CD11c<sup>+</sup> dendritic cells, (E) Ly6G<sup>+</sup> neutrophils, or (F) CD3<sup>+</sup> T cells (n=6 for A, C–F). **G**, Transforming growth factor (TGF)-β and (H) interleukin (IL)-1β levels measured in the supernatant of platelet-splenocyte coincubation (24 h; n=6). \**P*<0.05.

age of 17 weeks (baseline) and were repeated every fifth day for 12 weeks. At study end, plasma cholesterol levels of all groups had increased when compared with plasma cholesterol levels of the baseline group, but no differences were observed between the platelet- and vehicle-injected animals (baseline, 318.5±53.2 mg/dL versus *Apoe*<sup>-/-</sup> 523.7±27.6 mg/dL versus *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> 532.3±31.2 mg/dL versus vehicle 469.7±40.8 mg/dL; n=11). After the injection, blood leukocyte populations were analyzed by flow cytometry. No differences in total leukocytes (CD45<sup>+</sup>), monocytes (CD11b<sup>+</sup>), T cells (CD3<sup>+</sup>), or B cells (B220<sup>+</sup>) were observed. However, a relative decrease in the amount of Ly6G<sup>+</sup> neutrophils was detected in the animals injected with *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets (Figure IV in the online-only Data Supplement).

As expected, *Apoe*<sup>-/-</sup> mice injected with activated *Apoe*<sup>-/-</sup> platelets displayed a 2-fold increase in plaque area in the aortic arch and its major branch points when compared with vehicle-treated animals (Figure 5A and 5B). Of note, the proatherogenic effect of platelet-infusion was irrespective of the *Apoe* genotype (Figure V in the online-only Data Supplement). Remarkably, this platelet-induced acceleration of atherosclerosis was prevented in *Apoe*<sup>-/-</sup> mice injected with *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup>

platelets. Analysis of plaque size in the ascending aorta corroborated the central role of CD40 in platelet-induced plaque progression (Figure 5C).

The increase in plaque size in *Apoe*<sup>-/-</sup> animals injected with *Apoe*<sup>-/-</sup> platelets was associated with more inflammatory and advanced fibrotic plaque characteristics, as reflected by the high amount of fibrous cap atheroma (as classified by Virmani et al<sup>43</sup>) in this experimental group (Figure 5D–5E). *Apoe*<sup>-/-</sup> mice injected with *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets only displayed an initial plaque phenotype corresponding to intimal xanthomas, characterized by focal accumulations of foam cells and pathological intimal thickening where true necrosis is not yet apparent. This initial lesion phenotype was reflected by the reduction in collagen and lipid core size in the plaques of *Apoe*<sup>-/-</sup> mice injected with *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets (Figure 5F and 5G). Furthermore, plaques of mice injected with *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets exhibited a significant decreased number of Mac-3<sup>+</sup> macrophages, Ly6G<sup>+</sup> neutrophils, and a trend toward a decreased T-cell content and reduced expression of caspase-3 (Figure 5H–5K). Overall, these data clearly demonstrate that platelet CD40 contributes to atherogenesis in *Apoe*<sup>-/-</sup> mice.



**Figure 3.** Platelet cluster of differentiation 40 (CD40) recruits leukocytes into thrombi. Leukocyte recruitment into collagen-induced, thrombin-activated, *Apoe*<sup>-/-</sup>, *Selp*<sup>-/-</sup>*Apoe*<sup>-/-</sup>, *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup>, and *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelet aggregates. **A**, Numbers of leukocytes adhering to the platelet thrombi per field (n=6, \**P*<0.05). Depicted is the average of 25 counted microscopic fields. **B**, Representative microscopic images. Scale bar, 20  $\mu$ m. **C**, Thrombus density expressed as relative platelet-covered area. Two-photon microscopic analysis of percentage of **(D)** CD11b<sup>+</sup>Ly6G<sup>+</sup> and **(E)** CD11b<sup>+</sup>Ly6G<sup>+</sup> leukocytes recruited to the thrombi generated from platelets of the respective mice. **F**, Representative 2-photon image (Ly6G-FITC, CD11b-PE-Cy7, Annexin V-PE). Scale bar, 20  $\mu$ m.

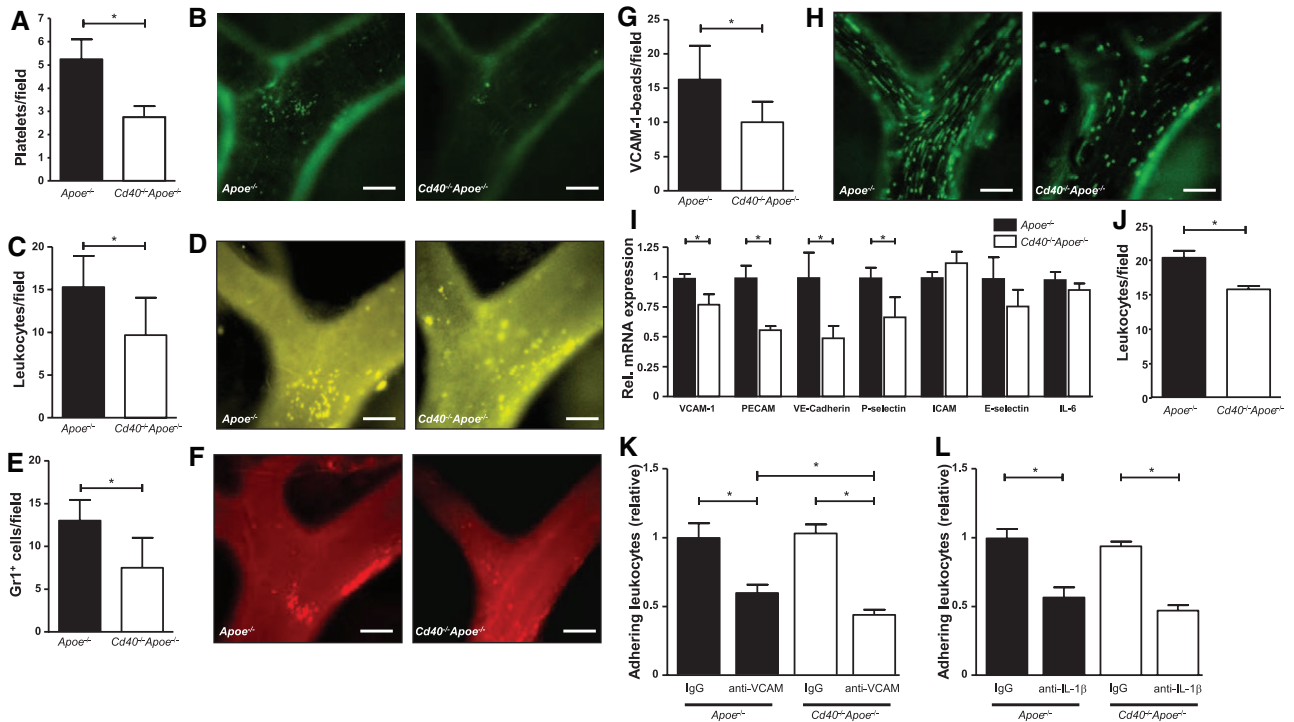
## Discussion

The capacity of platelets to promote inflammation is increasingly recognized, but the full potential of the platelet as immune cell is still enigmatic. Here, we identified platelet-CD40 as an important regulator of inflammatory processes that promote atherosclerosis.

One of the mechanisms how platelets exert inflammatory actions is through the heterotypic interaction with different immune cells promoting their recruitment and activation.<sup>44</sup> Platelets can form these heterotypic complexes via membrane receptors such as P-selectin, CD40L, and  $\alpha$ Ib $\beta$ 3 that bind PSGL, CD40, and CD11b, respectively, on the leukocyte.<sup>2,36</sup> Elevated levels of circulating platelet-leukocyte aggregates have been reported in several cardiovascular diseases and suggested to contribute to disease pathology. Increased levels of platelet-monocyte aggregates are found in patients with acute myocardial infarction and can therefore be seen as an early marker of acute myocardial infarction.<sup>45</sup> Selectively blocking platelet-monocyte interactions is currently under evaluation in clinical trials. Inclacumab, an anti-P-selectin antibody,

hinders the formation of platelet-monocyte aggregates and thereby prevents the inflammatory burst of these interactions.<sup>46</sup> Likewise, acute coronary syndromes are characterized by intense neutrophil activation. Recent studies report that this activation is induced by platelet-P selectin interactions inducing complete myeloperoxidase depletion in neutrophils.<sup>47</sup> Furthermore, in vitro studies revealed that platelets control the recruitment, activation, differentiation, and cytokine production of different CD4<sup>+</sup> T-cell subsets.<sup>48</sup> We recently showed in vivo that platelet CD40L mediates the reduction of circulating Treg cells, thereby promoting the development of atherosclerosis.<sup>36</sup>

When injecting platelets deficient for CD40, no differences in counts of total leukocyte, monocytes, T cell, or B cells were noticed, whereas a relative decrease in the amount of Ly6G<sup>+</sup> neutrophils when compared with wild-type platelet-injected mice was observed. Until now, we have no clear explanation how the relative reduction in circulating neutrophils is induced. It is conceivable that injection of thrombin-activated platelets deficient in CD40 may result in transiently less acute



**Figure 4.** Platelet cluster of differentiation 40 (CD40) deficiency reduces endothelial activation and neutrophil recruitment. **A** and **B**, Intravital microscopic analysis of platelet adhesion to endothelium in the carotid artery of *Apoe*<sup>-/-</sup> mice treated with tumor necrosis factor (TNF)- $\alpha$  (500 ng, IP) for 30 min and subsequently infused with thrombin-activated platelets (0.5 nmol/L, 15 min;  $3 \times 10^7/20$  g) isolated from *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice ( $n=4$ ). **C–H**, Intravital microscopy of carotid arteries from *Apoe*<sup>-/-</sup> mice fed a Western-type diet for 4 wk and injected with *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets every fifth day. **C** and **D**, Interactions of leukocytes with the carotid artery visualized by rhodamine injection. **E** and **F**, Inflammatory monocytes and neutrophils were visualized with an antibody to Gr1. **G** and **H**, Luminal arrest of fluorescent beads conjugated with antibody to vascular cell adhesion molecule (VCAM)-1 ( $n=6$ ; scale bar, 100  $\mu$ m for **B**, **D**, **F**, and **H**). **I**, Quantitative polymerase chain reaction to determine mRNA expression of various adhesion molecules and inflammatory mediators in endothelial cells (SVEC4-10) coincubated for 12 h with thrombin-activated platelets from *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice. Data are expressed relative to the control group ( $n=4$ ). **J**, Confluent endothelial cells (SVEC4-10), stimulated with TNF- $\alpha$  (10 ng/mL; 4 h) were exposed in flow chambers to perfusion with thrombin-activated platelets from *Apoe*<sup>-/-</sup> or *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice for 20 min followed by perfusion with calcein-labeled leukocytes (RAW 264.7). Adherent cells per field of view were counted. Similar leukocyte adhesion flow chamber assays performed in the presence of (**K**) anti-VCAM and (**L**) anti-IL-1 $\beta$  antibodies or the respective isotype-matched controls ( $n=4$ , 8 replicate fields; for **I–K**). \* $P<0.05$ . ICAM indicates intercellular adhesion molecule; and PECAM, platelet endothelial cell adhesion molecule.

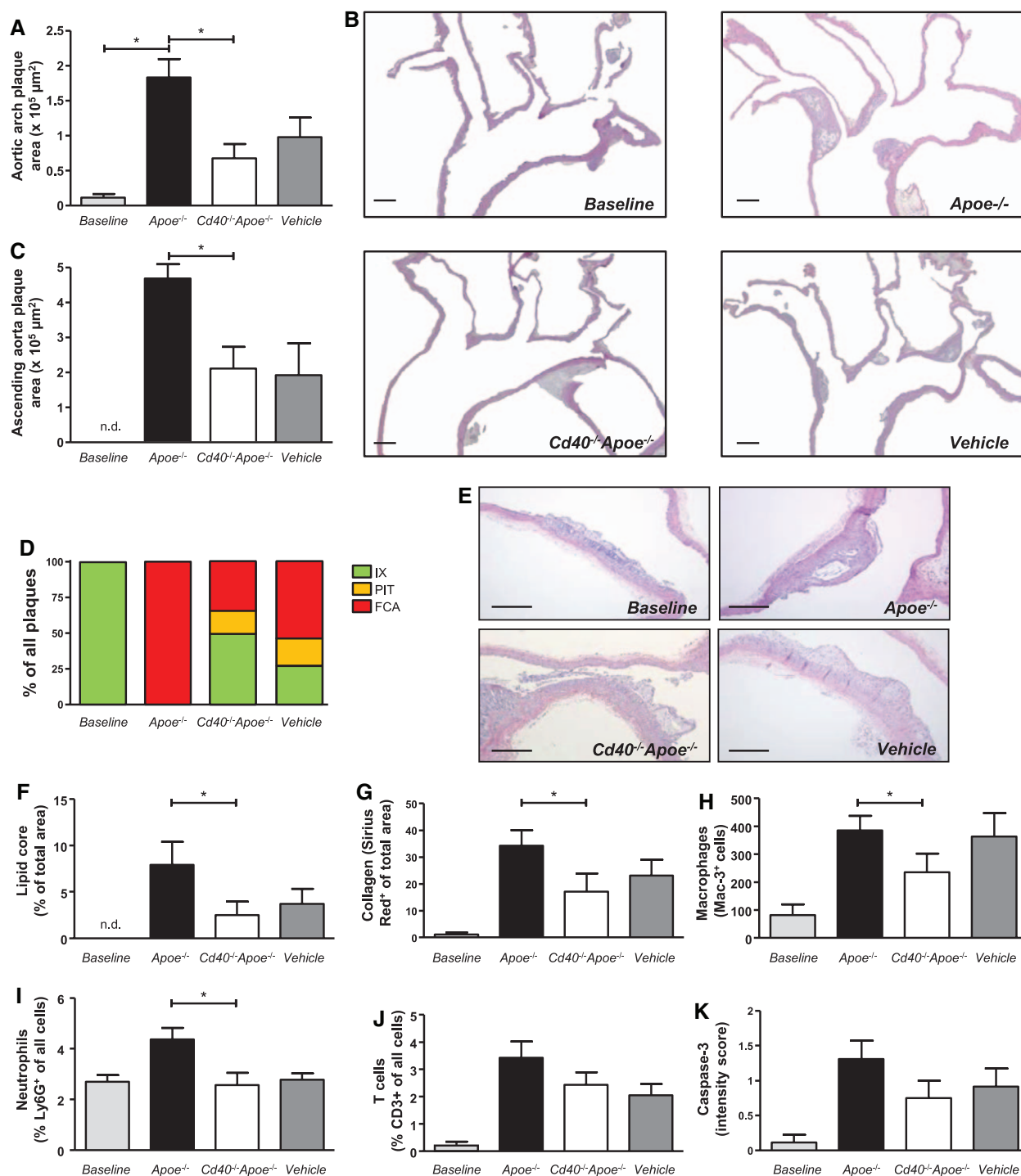
inflammatory stimuli than injection of thrombin-activated wild-type platelets, resulting in lower number of neutrophils in the circulation. We assume that the systemic reduction in neutrophils may affect their recruitment rate. However, this effect may play only a minor role in total leukocyte recruitment, whereas the predominant effect of platelet CD40 on leukocyte recruitment is most likely mediated via EC activation.

Here, we observed that platelet CD40 can also bind leukocytes and form platelet–leukocyte aggregates. Platelet CD40 particularly bound neutrophils and monocytes, dendritic cells, but not T cells. CD40-dependent platelet–leukocyte cross talk promoted the release of IL-1 $\beta$ , a major mediator of platelet-induced activation of the endothelium by inducing endothelial adhesion molecule expression (VCAM-1 and intercellular adhesion molecule-1) and cytokine production (IL-6 and IL-8).<sup>49,50</sup> In addition, we show that platelet CD40 has an important role in promoting endothelial activation and attracting more monocytes and neutrophils toward the site of inflammation.

Not only single platelets stimulate inflammation but also platelet aggregates are considered to be proinflammatory. During the time course of atherosclerosis, platelet aggregates continuously form on the endothelium. Because these aggregates consist of activated platelets releasing their

granule content and inflammatory mediators, it is conceivable that this instigated inflammatory milieu enhances leukocyte recruitment, and thereby aggravates atherosclerosis. We here reveal that recruitment of leukocytes into platelet aggregates is strongly mediated by platelet CD40, as well as by platelet CD40L and P-selectin. Autopsy studies suggest that during life, the vasculature undergoes numerous plaque ruptures with consequent (nonocclusive) thrombus formation. These thrombi are resolved without causing any symptoms.<sup>42</sup> Because frequently occurring (silent) thrombi induce rapid progression of atherosclerosis, we consider CD40-mediated leukocyte recruitment into thrombi as another important mechanism in the repertoire of platelet immunity.<sup>42</sup>

Unlike its potential to induce inflammation, we could not find a role for platelet CD40 in platelet–platelet interactions when the major platelet receptors GPVI (convulxin), purinergic receptor class 2, subclass Y1/Y2 (ADP), and protease-activated receptor-3/4 (thrombin) were activated. These results are in contrast with the previous report by Inwald et al,<sup>13</sup> who reported that ligation of platelet CD40 caused increased P-selectin expression and the classical morphological changes associated with platelet activation, yet did not result in Ca<sup>2+</sup> release. However, instead of CD40 deficiency, these authors



**Figure 5.** Platelet cluster of differentiation 40 (CD40) expression mediates platelet-induced atherosclerosis. Seventeen-week-old *Apoe*<sup>-/-</sup> mice (n=11) were injected for 12 wk with thrombin-activated *Apoe*<sup>-/-</sup> platelets, *Cd40*<sup>-/-</sup>*Apoe*<sup>-/-</sup> platelets, or vehicle and euthanized at the age of 29 wk. Baseline animals (17 wk) were included. **A**, Plaque area (μm<sup>2</sup>) in the aortic arch and main branch points (n=11). **B**, Representative longitudinal images of the aortic arch and branch points (hematoxylin-eosin [HE] staining). **C**, Plaque area (μm<sup>2</sup>) in the aortic root (n=5). **D**, Lesions of the aortic arch and branch points were classified according to Virmani guidelines. **E**, Representative longitudinal images of the brachiocephalic artery (HE staining). **F–K**, Histological analysis of the aortic arch and its major branch points. **F**, Lipid core content measured by Oil Red O. **G**, Plaque collagen content measured as Sirius red<sup>+</sup> staining. **H**, Absolute numbers of Mac-3<sup>+</sup> macrophages per section. **I**, Relative frequency of Ly6G<sup>+</sup> neutrophils. **J**, Percentage of CD3<sup>+</sup> T cells. **K**, Cleaved caspase-3 staining was graded from 0 (not present) to 3 (highly present; n=11 for **F–K**). Scale bar, 200 μm; \**P*<0.05. FCA indicates fibrous cap atheroma; IX, intimal xanthoma; n.d., not detected; and PIT, pathological intimal thickening.

used a recombinant form of sCD40L to induce platelet activation, whereas we used thrombin-induced platelet activation.<sup>13</sup> Recently, we have identified that CD40L and CD40 have, in

part, different roles in collagen-induced platelet aggregation and thrombus growth.<sup>39</sup> Although CD40 deficiency did impair GP VI-induced platelet aggregation on atherosclerotic plaque



material or on collagen, CD40 only had a modest role in thrombus formation, whereas platelet CD40L is important in collagen-induced thrombus formation and growth.<sup>39</sup> Still, platelet CD40 signaling remains only poorly understood. Intracellular communication may involve the CD40-tumor necrosis factor–associated factor signaling pathway, as is known in leukocytes.<sup>19</sup> Of note, it was reported that sCD40L exacerbates platelet activation and aggregation through a CD40-dependent tumor necrosis factor–associated factor 2 mechanism.<sup>51</sup>

Taken together, inhibition of platelet CD40 represents an attractive therapeutic target. Because antibody treatment of its counterpart CD40L caused thromboembolic events, clinical trials were halted.<sup>38,52</sup> Hence, antagonizing CD40 provides a viable alternative offering many advantages. During the past decade, researchers have aimed to develop CD40 as a treatment for cancer because of its inflammatory capacity and overexpression in certain tumors. The recently developed experimental CD40-antagonizing antibody is currently extensively tested in cancer research where it is thought to block proliferation of CD40<sup>+</sup> malignant cells.<sup>53</sup> A safety trial with another anti-CD40-antagonizing antibody, ch5D12, showed a reduction in inflammatory activity in biopsies of patients having patients with Crohn disease.<sup>54</sup>

With the results of the current study, we provide a novel therapeutic target in cardiovascular medicine acting at the critical interface between inflammation, thrombosis, and atherosclerosis. Selectively blocking platelet CD40 will impede platelet-induced leukocyte recruitment and endothelium activation eventually reducing formation and complication of atherosclerosis.

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### Disclosures

None.

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## Significance

Besides their obvious thrombogenic function in vascular pathologies, platelets can also initiate and sustain inflammatory processes, predominantly by triggering leukocyte recruitment toward the core of the inflammation. Cluster of differentiation 40 (CD40) and its ligand CD40L, present on not only many immune cells but also on platelets, are well-known costimulatory molecules that mediate both thrombotic and immune reactions. In this study, we investigated the inflammatory power of platelet-specific expression of CD40, an integral membrane protein of the tumor necrosis factor receptor family. Our data reveal an important role for platelet CD40 in the progression of atherosclerosis. Platelet CD40 amplifies immune responses by activating leukocytes and endothelial cells, thereby stimulating the recruitment of inflammatory cells toward the atherosclerotic plaque. Therefore, selectively blocking platelet CD40 holds potential for atherosclerosis treatment and might be beneficial for other inflammatory diseases as well.